The *Synechocystis* sp PCC 6803 Oxa1 Homolog Is Essential for Membrane Integration of Reaction Center Precursor Protein pD1

Friedrich Ossenbühl, Masami Inaba-Sulpice, Jörg Meurer, Jürgen Soll, and Lutz A. Eichacker

*Department for Molecular Botany, University Ulm, D-89069 Ulm, Germany*

*Department for Biology I, Botany, Ludwig-Maximilians-University, D-80638 Munich, Germany*

*Max-Planck-Institute of Molecular Plant Physiology, D-14476 Potsdam-Golm, Germany*

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**INTRODUCTION**

It is generally accepted that chloroplasts of higher plants descended from cyanobacteria (Hermann, 1997; McFadden and van Dooren, 2004). During endosymbiosis, most of the cyanobacterial genes were transferred to the nucleus of the host cell. As a result, most of the chloroplast-localized proteins are nucleus-encoded and have to be imported (Abdallah et al., 2000; Martin et al., 2002). Upon import, the proteins are targeted to the soluble (stroma and the thylakoid lumen) and membrane (inner envelope and thylakoid membrane) compartments of the chloroplast. To date, four different pathways (the spontaneous and the bacteria-related cpSec, cpTAT/ΔpH, and cpSRP pathways) have been described for the targeting of imported proteins into and across the thylakoid membrane, which require specific sets of protein factors and energy sources (Keegstra and Cline, 1999; Eichacker and Henry, 2001; Mori and Cline, 2001; Schleiff and Klösgen, 2001).

By contrast, little is known about the targeting of plastid-encoded proteins to the thylakoid membrane. Recent results suggest that targeting, membrane export, and assembly of the D1 reaction center protein of photosystem II (PSII) might be performed by components of the cpSRP and cpSec pathways: cpSRP54, Alb3p, and cpSecY (Nilsson et al., 1999; Zhang et al., 2001). Specifically, cpSRP54 was found to interact early with the nascent D1 protein (D1 fragments smaller than 17 kD) (Nilsson et al., 1999), whereas nascent D1 fragments between 17 and 25 kD were found in interaction with the translocase cpSecY (Zhang et al., 2001). Recently, Alb3.1p of *Chlamydomonas reinhardtii* was shown to selectively interact with the full-length reaction center protein (RC) D1 during the assembly of D1 into PSII (Ossenbühl et al., 2004). Alb3p belongs to a widespread protein family, the Oxa1p/Alb3/YidC family. Members of this protein family are found in bacteria, mitochondria, and chloroplasts. They constitute a group of evolutionarily conserved proteins that appear to be involved in the integration and/or assembly of membrane protein complexes. The mitochondrial Oxa1p and the bacterial YidCp are the best analyzed examples (Kühn et al., 2003). Oxa1p is localized at the inner membrane of mitochondria and facilitates the insertion of both nucleus- and mitochondria-encoded proteins both in connection with the Sec translocase and on its own. Recent models proposed that the YidC protein might also be required for the assembly of inserted membrane proteins by clearing the SecYEG translocase (Chen et al., 2002). Interestingly, the ΔyidC mutant of *E. coli* can be complemented with ALB3 from *Arabidopsis thaliana*, thus indicating a common function (Jiang et al., 2002). The protein ALB3p was originally identified by complementation of an albino3 (alb3) mutant of *Arabidopsis* (Sundberg et al., 1997). The phenotype of the alb3 mutant was described as very severe with strongly reduced thylakoid membranes, suggesting an additional function of Alb3 besides the described integration of light-harvesting complex proteins (LHCPs) (Moore et al., 2000, 2003).
In C. reinhardtii, two nuclear genes encoding ALB3p homologs, Alb3.1p and Alb3.2p, were identified by complementation of a nuclear mutant of the Alb3.1 gene, ac29 (Beliafiore et al., 2002; Göhre et al., 2006). The phenotype of ac29, which accumulates ~10% LHCPs compared with the wild type, revealed a less pronounced decrease in PSII than the alb3 phenotype in Arabidopsis. In addition to the reduced LHCP levels, a retarded D1 assembly into PSII was found, suggesting that Alb3.1p is involved in the integration and assembly of both nucleus- and plastid-encoded proteins in the thylakoid membranes (Ossenbühl et al., 2004). In the unicellular cyanobacterium Synechocystis sp PCC 6803 (hereafter called Synechocystis), Slr1471p (encoded by the slr1471 gene) was found to be involved in cell division by an unknown mechanism. However, the protein Slr1471p lacks an N-terminal receptor domain, which in Arabidopsis was implicated in organellar division (Fulgosi et al., 2002). Because only the single gene slr1471 was found in all cyanobacterial genome projects, Slr1471p may have an even broader division by an unknown mechanism. However, the protein Slr1471p lacks an N-terminal receptor domain, which in Arabidopsis was implicated in organellar division (Fulgosi et al., 2002). Because only the single gene slr1471 was found in all cyanobacterial genome projects, Slr1471p may have an even broader spectrum of substrates and modes of operation than in chloroplasts. This idea is supported by the fact that it is by now not possible to fully segregate a deletion mutant of slr1471. Previous results with partially segregated knockdown mutants suggested that Slr1471p plays a role not only in cell division but in membrane biogenesis as well (Spence et al., 2004). Here, we show that Slr1471p function is significantly impaired by the fusion of green fluorescent protein (GFP) to its C terminus. Specifically, mutant light sensitivity was found to be mediated by an alteration in the redox potential of reaction center quinones QA− and QB−, leading to the photoinhibition of PSII and a concomitant decrease in the mutant growth rate. These results suggest that Slr1471p is important for the de novo assembly of the D1 precursor protein into the PSII reaction center.

RESULTS

Fusion of GFP to the C Terminus of Slr1471p Causes a Light-Sensitive Growth Defect

To characterize the function of Slr1471p in cyanobacteria, we fused the C terminus of Slr1471p with the globular and soluble GFP (Figure 1A). Replacement of all copies of the wild-type chromosome in the mutant was verified by PCR and expression of the fusion protein by gel blot analysis using antibodies against Slr1471p (Figure 1B). Surprisingly, during analysis of the photoautotrophic growth of slr1471-gfp cells, we found that the growth of mutant cells grown under a light intensity of 10 μmol·m⁻²·s⁻¹ was impaired when light intensities were shifted to 20, 40, and 80 μmol·m⁻²·s⁻¹ (wild-type cells versus slr1471-gfp cells; Figure 1C). When light intensities were increased, wild-type cells responded with growth to higher densities (cell densities in different light conditions for the wild type were as follows: 20 μmol·m⁻²·s⁻¹, 0.292 × 10⁻⁶ OD₇₃₀/h; 40 μmol·m⁻²·s⁻¹, 0.958 × 10⁻⁶ OD₇₃₀/h; 80 μmol·m⁻²·s⁻¹, 1.472 × 10⁻⁶ OD₇₃₀/h), whereas slr1471-gfp cells grew to lower densities (cell densities in different light conditions for slr1471-gfp were as follows: 20 μmol·m⁻²·s⁻¹, 0.007 × 10⁻⁶ OD₇₃₀/h; 40 μmol·m⁻²·s⁻¹, 0.000 × 10⁻⁶ OD₇₃₀/h; 80 μmol·m⁻²·s⁻¹, 0.167 × 10⁻⁶ OD₇₃₀/h).

For segregation of the mutant, GFP was inserted together with a kanamycin resistance (KmR) gene cartridge. To ensure that the light depression of growth in slr1471-gfp was not caused by KmR, we generated the mutant slr1471-KmR (Figure 1A). We found that the growth of slr1471-KmR cells at different light intensities was comparable to that of the wild type (Figure 1C) (cell densities in different light conditions for slr1471-KmR were as follows: 20 μmol·m⁻²·s⁻¹, 0.319 × 10⁻⁶ OD₇₃₀/h; 40 μmol·m⁻²·s⁻¹, 0.972 × 10⁻⁶ OD₇₃₀/h; 80 μmol·m⁻²·s⁻¹, 1.486 × 10⁻⁶ OD₇₃₀/h). We conclude that the fusion of GFP specifically alters the function of Slr1471p, causing the inhibition of photoautotrophic growth at higher light intensity.

Interestingly, absorption spectra of slr1471-gfp cells grown at 80 μmol·m⁻²·s⁻¹ indicated that at this increased light intensity,
pigmentation was also selectively changed in the mutant (Figure 1C). To compare the absorption spectra of the wild type, slr1471-gfp, and slr1471-KmR grown at low (20 μmol m⁻² s⁻¹) and high (80 μmol m⁻² s⁻¹) light intensity, the spectra of all three strains shown in Figure 1C were normalized to the highest peak at 436 nm (Figure 2A). slr1471-KmR spectra revealed a small decrease in carotenoid content only at the highest light intensity. In slr1471-gfp, overall small decreases of carotenoid (470 to 500 nm), phycobilin (550 to 650 nm), and chlorophyll (670 nm) contents correlated with decreased growth at low light intensity; however, at increased light intensity, pigment contents were selectively reduced, in particular carotenoids and phycobilins (Figure 2A). We also compared the relative amounts of the major thylakoid membrane complexes and of phycobilins bound to phycobilisomes in all three strains grown under high light by immunoblot analysis using antibodies against central subunits (Figure 2B) and by fluorescence and visible scans of the phycobiliproteins on the gel (Figure 2C). These analyses revealed no significant differences in the amount of the major thylakoid membrane complexes PSI, PSII, ATP synthase, and cytochrome b_{6f} between the wild type, slr1471-gfp, and slr1471-KmR (Figure 2B). By contrast, phycobiliproteins showed a reduced fluorescence signal in slr1471-gfp compared with the wild type and slr1471-KmR (Figure 2C), corroborating the reduced amount of phycobilins in slr1471-gfp (Figure 2A). These results indicate in particular that the accumulation of functional proteins from the light-harvesting antenna was affected. Therefore, we analyzed the fluorescence properties of the different antenna systems of PSI and PSII by 77K fluorescence spectroscopy using excitation wavelengths at 440 nm (chlorophyll a) (Figure 3A) and 570 nm (phycobilisomes) (Figure 3B), respectively.

Under both low- and high-light conditions, slr1471-KmR cells showed no significant change in PSI antenna fluorescence from chlorophyll a (excitation wavelength, 440 nm) compared with wild-type cells. In addition, low-light-grown slr1471-gfp showed 77K fluorescence spectra comparable to wild type cells, with emission peaks at 686, 695, and 725 nm corresponding to CP43, CP47, and PSI, respectively (Figure 3A; see Supplemental Figure 1 online; Shen and Vermaas, 1994). However, high-light-grown cells of slr1471-gfp showed a selectively reduced fluorescence emission of CP47 at 695 nm and a shifted PSI emission at 723.5 nm (Figure 3A). These results were confirmed by the 77K fluorescence spectra obtained after excitation at 570 nm. Besides the described PSII and PSI peaks, emission spectra showed additional peaks at 650 and 688 nm caused by the fluorescence of the phycobilisome pigments phycocyanin and allophycocyanin, respectively (Figure 3B; see Supplemental Figure 2 online). As after excitation at 440 nm, the emission spectra of low-light-grown slr1471-gfp and slr1471-KmR cells excited at 570 nm were comparable to the wild-type spectra, whereas high-light-grown cells of slr1471-gfp showed a selectively and strongly reduced fluorescence emission of CP47 at 695 nm and a shifted PSI emission at 723.5 nm (Figure 3B). In addition, the phycocyanin emission peak of high-light-grown slr1471-gfp appeared at 650 nm instead of 650 nm in high-light-grown wild-type and slr1471-KmR cells (Figure 3B; see Supplemental Figure 2 online). PSI fluorescence in high-light-grown slr1471-gfp was reduced to half compared with the wild type, even though the amount of PSI proteins was not changed (Figures 2B and 2C). Furthermore, we detected a blue shift of the PSI fluorescence by 1.5 nm in high-light-grown slr1471-gfp (Figures 3A and 3B).

We conclude that in slr1471-gfp under high-light conditions, the quantum transfer between phycobilisomes, which operate as the outer and inner antenna of the reaction center core complexes, may be affected as a result of reduced amounts of phycobilisomes. This was corroborated by our finding that the protein contents of the major thylakoid membrane complexes in the wild type, slr1471-gfp, and slr1471-KmR grown under different light did not reveal any significant differences in PSI, PSII, cytochrome b_{6f},
and ATPase content by immunoblot analysis but showed a decreased amount of phycobiliproteins (Figures 2B and 2C). Together, these data indicate an altered pigmentation in slr1471-gfp that may result in changes of quantum or electron transfer.

**Electron Transfer within PSII of slr1471-gfp Is Altered**

To reveal whether changes in quantum transfer within PSII and PSI or electron transfer between the photosystems were responsible for the light sensitivity of slr1471-gfp, we performed oxygen evolution and pulse amplitude–modulated room temperature chlorophyll fluorescence (PAM) measurements. For oxygen evolution, strains were grown and measured at 20 and 80 μmol·m⁻²·s⁻¹. The relative photosynthetic yield remained unchanged at low light; however, at the increased light intensity, in slr1471-gfp only ~60% could be obtained (Table 1). For PAM, strains were grown under low- and high-light conditions, and after dark adaptation for 2 min, measurements were performed as described in Methods with an actinic light intensity of 20 μmol·m⁻²·s⁻¹. Low-light-grown cells showed a maximum photochemical efficiency of PSII in the dark-adapted state (Fv/Fm) of 0.43 ± 0.02 for both the wild type and slr1471-KmR and of 0.40 ± 0.02 for slr1471-gfp (see Supplemental Figure 3 online). Under actinic light, photochemical quenching was comparable in all three strains grown under low light (see Supplemental Figure 3 online). When grown under high light, the wild type and slr1471-KmR performed similarly, whereas slr1471-gfp displayed clear differences (see Supplemental Figure 3 online). The Fv/Fm was decreased to 0.20 ± 0.02 and the actinic light–induced quenching resulted in a steady state chlorophyll fluorescence lower than the initial (minimum) PSII fluorescence in the dark-adapted state (F₀). Furthermore, the chlorophyll fluorescence did not recover to F₀ even after 15 min of dark incubation. This indicated that under actinic light, the functionality of PSII was decreased as a result of chlorophyll a bleaching.

To confirm that the decrease of chlorophyll fluorescence is caused by a malfunction of PSII, cells were grown under low light and the chlorophyll fluorescence was compared under actinic light intensities of 20 and 200 μmol·m⁻²·s⁻¹ in the presence of the herbicide DCMU. Under these experimental conditions, chlorophyll fluorescence was expected to remain high under actinic light, because DCMU blocks electron transport between PSII and cytochrome b₆f (Figure 4). Under low actinic light, the wild type, slr1471-gfp, and slr1471-KmR revealed no significant differences (Figure 4). However, high actinic light treatments led to a decrease of the steady state chlorophyll a fluorescence in slr1471-gfp only, demonstrating chlorophyll a bleaching. Switching off the high actinic light again for slr1471-gfp demonstrated that the dark chlorophyll fluorescence F₀ value remained below the starting F₀ value, whereas the wild type and slr1471-KmR remained at values above the initial F₀ level and also comparable to wild-type and slr1471-KmR values after low actinic illumination (Figure 4). In addition, F₀ levels in slr1471-gfp cells did not recover after high actinic light treatment even after 20 min of dark incubation. Because DCMU blocks the Q₀ binding site in D1, inhibiting electron efflux from Q₀, we concluded that rapid

**Table 1. Oxygen Evolution of Wild-Type, slr1471-gfp, and slr1471-KmR Cells Grown and Measured under Different Light Conditions**

<table>
<thead>
<tr>
<th>Strain</th>
<th>20 μmol·m⁻²·s⁻¹</th>
<th>Relative Oxygen Evolution</th>
<th>80 μmol·m⁻²·s⁻¹</th>
<th>Relative Oxygen Evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol Oxygen·h⁻¹·μg⁻¹</td>
<td></td>
<td>μmol Oxygen·h⁻¹·μg⁻¹</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>97 ± 11</td>
<td>100</td>
<td>165 ± 22</td>
<td>100</td>
</tr>
<tr>
<td>slr1471-gfp</td>
<td>99 ± 14</td>
<td>102</td>
<td>102 ± 21</td>
<td>62</td>
</tr>
<tr>
<td>slr1471-KmR</td>
<td>90 ± 12</td>
<td>93</td>
<td>167 ± 33</td>
<td>101</td>
</tr>
</tbody>
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chlorophyll a bleaching in slr1471-gfp resulted from an altered Q_A function or redox potential.

**Redox Potentials of Q_A^- and Q_B^- Are Altered in slr1471-gfp**

To measure the Q_A^- and Q_B^- redox potentials in wild-type, slr1471-gfp, and slr1471-KmR cells, we performed thermoluminescence measurements (Figure 5; see Supplemental Figure 4 online). No significant changes were found for the wild type and slr1471-KmR. In both cases, the highest thermoluminescence emission revealed a B-band emission maximum at 34.2°C for recombinations from the S_2^-P_680Q_A^- state, indicating an unaltered redox potential for Q_A^- . However, for slr1471-gfp, B-band emission was extended to lower temperatures, with a maximum at 31.3°C, indicating that the redox potential for Q_A^- is decreased in the mutant (Figure 5, -DCMU). Fitting of the thermoluminescence spectra revealed the described B-bands for the wild type and slr1471-KmR, but besides the described B-band at 31.3°C, there was an additional significantly smaller peak for slr1471-gfp with an emission maximum temperature of 21.3°C, indicating the emission of a small pool of S_2^-P_680Q_A^- states.

In the presence of DCMU, a competitive inhibitor of Q_B, thermoluminescence measurements with wild-type and slr1471-KmR cells resulted in a Q-band emission maximum of 8.0°C for charge recombination from the S_2^-P_680Q_A^- state. By contrast, in slr1471-gfp cells, an increased Q-band emission maximum at 15.7°C for the S_2^-P_680Q_A^- state indicated an increase in the redox potential of the Q_A^- state (Figure 5, +DCMU). In addition to the Q-band, all spectra showed C-bands at ~47°C, which seemed similar in all three strains. We performed a curve fit again, revealing two already described peaks at 8.0 and 47.1°C for both the wild type and slr1471-KmR (Figure 5). However, curve fitting of the slr1471-gfp thermoluminescence spectrum in the presence of DCMU showed two peaks at 14.5 and 21.8°C, which appeared merged in the peak at 15.7°C of the original curve, as well as a peak at 47.1°C corresponding to the wild type and slr1471-KmR. It is noteworthy that the intensity of the peaks at 14.5 and 21.8°C was nearly identical, indicating the presence of two pools of S_2^-P_680Q_A^- states in slr1471-gfp. We conclude that in slr1471-gfp, the redox potentials of Q_A^- and Q_B^- are altered, leading to the pronounced light sensitivity and photo-inhibition of PSII. These results clearly indicate an altered structure of the PSII reaction center protein D1 and most likely also D2.

**D1 Integration into Thylakoid Membranes Is Impaired in slr1471-gfp**

For photosynthetic function, Q_A and Q_B have to be assembled with PSII reaction center proteins D2 and D1, respectively. Therefore, we addressed the function of Slr1471p for membrane integration or assembly of D1 and/or D2 by in vivo labeling analysis of wild-type, slr1471-gfp, and slr1471-KmR cells at 30°C and 20 μmol m^{-2} s^{-1}. Cells grown under low light were incubated for 15 and 75 min in radiolabeling pulse reactions or were pulse-labeled for 15 min followed by a 60-min chase with L-Met. Isolated and solubilized total membranes were then separated on SDS gels (Figure 6A). In all three strains, the PSII reaction center proteins D1 and D2 were labeled; but in slr1471-gfp, a strong signal with a molecular mass close to that of the D2
protein appeared. To differentiate between radiolabel accumulation in D2 and precursor D1 (pD1), we immunoprecipitated D1 using a D1-specific antibody after denaturing of the isolated membranes with SDS. The results revealed that D1 accumulated dominantly in the pD1 form but also as a mature D1 in \textit{slr1471-gfp} (Figure 6B). Interestingly, the presence of pD1 was independent of the light conditions but specific for \textit{slr1471-gfp} cells, indicating that in the mutant, PSII assembly was affected by an impaired processing of pD1. Because Q\textsubscript{A} and Q\textsubscript{B} are bound to transmembrane helices 4 and 5 of the PSII reaction center proteins D2 and D1, respectively, we speculated that either membrane integration or folding of D1 or D2, or both, could be altered in \textit{slr1471-gfp}. To study the integration status of D1, pD1, and D2 in the membrane, we incubated total membranes of all three strains with trypsin (Figure 6C).

The proteolytic pattern for \textit{slr1471-gfp} revealed four specific signals, which were either hardly detectable (Figure 6C, arrows) or present only as much weaker signals (Figure 6C, asterisks) in the wild type and \textit{slr1471-Km}\textsuperscript{R} (Figure 6C, lanes 6 and 7 compared with 2, 3, 10, and 11). Furthermore, during total membrane isolation, D1, pD1, and D2 were not released to the soluble fractions and could not be extracted from the membrane phase by carbonate. We conclude that accumulation of pD1 in the membrane phase in \textit{slr1471-gfp} increased the amount of degradation product most likely derived from pD1 that was not properly or not at all inserted into the membrane. Given that the precursor protein was not or not properly inserted, an assembly kinetics analysis should reveal difficulties during the assembly of PSII in \textit{slr1471-gfp} cells. Therefore, we performed two-dimensional blue native (BN)/SDS-PAGE with the isolated total membranes and analyzed the assembly kinetics of D1, pD1, and D2 in the three different strains. For PSII, six assembly states could be readily identified by radiolabeling of the D2 and D1 proteins in wild-type and \textit{slr1471-Km}\textsuperscript{R} cells [Figure 7, PSII\textsubscript{a}, RCC(2), RCC(1), RC47, RC\textsuperscript{c}, and RC]. In \textit{slr1471-gfp} cells, the assembly of PSII revealed a major difference (Figure 7). Assembly intermediates corresponding to reaction center complexes (RC and RC\textsuperscript{c}) were not detectable (Figure 7, white asterisks). This finding indicated that in \textit{slr1471-gfp}, processing of pD1 at the level of the RC assembly intermediate either was very rapid or the assembly step was omitted in the mutant. Furthermore, we noted that radiolabeled pD1 accumulated in \textit{slr1471-gfp} was present at apparently all molecular mass levels in the first BN dimension gel, indicating nonspecific aggregation of pD1.

Thus, \textit{slr1471-gfp} cells only partially accumulated or did not integrate pD1 at all. At least the last transmembrane helix (number 5) appeared not to be integrated properly during assembly, because processing of pD1 requires the C-terminal end to be exported into the thylakoid lumen. In addition, a mature D1 that assembled into PSII complexes displayed altered Q\textsubscript{A}\textsuperscript{−} and Q\textsubscript{B} redox potentials. These results suggested an impaired function of the Slr1471-GFP fusion protein for the integration and folding of D1 in the thylakoid membrane of \textit{Synechocystis}. This implies a direct interaction of Slr1471p with D1 and maybe an altered interaction of Slr1471-Gfp with D1. Therefore, we next analyzed whether Slr1471p interacts directly with the D1 protein in the wild-type strain. We used total membranes from the wild type and polyclonal antibodies specific for Slr1471p and D1 and investigated the SynOxa1–D1 interaction by coimmunoprecipitation.
(Figure 8A). After immunoprecipitation with D1 antibody, coimmunoprecipitated proteins were examined by gel blot analysis with antibodies against D1 and Slr1471p. Both proteins were clearly detectable (Figure 8A, column 2). Conversely, a stronger Strl471p and a weaker D1 gel blot signal were obtained for immunoprecipitation with an Slr1471p-specific antibody (Figure 8A, column 3). By contrast, immunoprecipitation with an antibody against AtpB could not effectively pull down D1 or Strl471p when analyzed with antibodies against D1 and Strl471p (Figure 8A, column 1). Therefore, we conclude that Strl471p interacts with D1 in Synechocystis.

We then performed coimmunoprecipitation using thylakoid membranes isolated from radioactively labeled wild-type and slr1471-gfp cells and a GFP-specific antibody coupled to microbeads (see Methods). As shown in Figure 8B, pD1 accumulating in slr1471-gfp (column 2) was specifically copurified with Strl471p but lacked mature D1 (column 4), whereas no D1 protein could be purified from the wild type (Figure 8B, column 3). These data suggest an interaction of mature D1 with Strl471p in the wild type, which seemed to be impaired in slr1471-gfp, because only pD1 interacts with Strl471p.

**DISCUSSION**

We found that fusion of GFP to the C terminus of Strl471p in Synechocystis resulted in light sensitivity and an altered growth rate of mutant slr1471-gfp. Analysis of PSII revealed an altered redox potential for QA \(- Q_{A}^{\cdot} \) and QB \(- Q_{B}^{\cdot} \). In combination with our finding that membrane integration and assembly of reaction center protein D1 was altered in the mutant, we conclude that Strl471p, the only member of the Alb3/Oxa1/YidC protein family in Synechocystis, operates as a membrane integral chaperone essential for the correct membrane integration, folding, and assembly of PSII reaction center precursor protein pD1.

**Figure 7.** Assembly of PSII Is Altered in slr1471-gfp.

Wild-type, slr1471-gfp, and slr1471-KmR cells (2 \times 10^6) were pulse- and pulse/chase-labeled with [35S]Met in vivo as described for Figure 5. Total membranes were separated by two-dimensional BN/SDS-PAGE. Complexes containing radioabeled proteins in the SDS-PAGE size range from 25 to 50 kD are presented in a molecular mass window as indicated in kilodaltons for BN-PAGE. Frames (dotted lines) highlight the positions of PSII assembly complexes [RC and RC', reaction center complexes of PSII including D1 and D2; RC47, reaction center complex of PSII including CP47; RCC(1) and RCC(2), monomeric and dimeric reaction center core complexes of PSII; PSII\(_{\text{vec}}\), PSII supercomplexes; “free protein,” unassembled protein solubilized from the membrane phase]. Radiolabeled proteins CP47, CP43, D2, pD1, intermediate D1 (iD1), and mature D1 (D1) are indicated. Asterisks mark areas of missing reaction center complexes in slr1471-gfp.

**Figure 8.** Interaction of Strl471p with D1.

(A) Total membranes from wild-type cells were solubilized with \(\beta\)-dodecylmaltoside. Immunoprecipitation was performed using antisera against AtpB (\(\alpha\)-AtpB), D1 (\(\alpha\)-D1), and Strl471p (\(\alpha\)-Strl471p). Immunoprecipitated proteins were analyzed by immunoblotting with antisera against D1 and Strl471p.

(B) Wild-type and slr1471-gfp cells were pulse-radiolabeled in vivo with [35S]Met for 15 min. Isolated total membranes (lanes 1 and 2) and isolated total membranes subjected to coimmunoprecipitation with an antibody against GFP (lanes 3 and 4) were separated by SDS-PAGE. Radiolabeled D1 and pD1 are indicated. Lanes 3 and 4 show a longer exposure of the same gel shown in lanes 1 and 2.
Analyzing the C-terminal fusion of GFP to Slr1471p, it was intriguing that Slr1471p not only acted as an assembly factor but also catalyzed the proper folding and correct integration of pD1. In higher plant chloroplasts, the D1 protein has been proposed to cotranslationally integrate into the thylakoid membrane or to cotranslationally assemble directly with preassembled reaction center proteins (Zhang et al., 2001). We show here that full-length D1 at least transiently interacts with Slr1471p. Hence, in Synechocystis, assisted membrane integration precedes the functional assembly of D1.

The accumulation of pD1 in slr1471-gfp was caused by the Slr1471-GFP fusion protein, which seemed to be unable or too slow to integrate and fold pD1 correctly. This can be concluded from the interaction of pD1, but not mature D1, with Slr1471-GFP. Interestingly, a pool of mature D1 was integrated completely into thylakoid membranes of slr1471-gfp, but these mature D1 proteins were not integrated and assembled properly into PSII complexes, as shown by altered redox potentials of QA and QB. We speculate that in the mutant, improperly integrated D1 may fail to correctly bind QB during folding, which in turn may alter the correct assembly of RC intermediates, leading to a structural alteration in the binding of QA to the D2 protein. Alternatively, Slr1471-GFP may also fail to integrate and fold D2 correctly into thylakoid membranes, leading to an altered binding of QA.

Interestingly, deconvolution of the original thermoluminescence curves measured in the presence of DCMU revealed two peaks of similar intensities at 14.5 and 21.8°C for the recombination of the S2-P680QA state in slr1471-gfp, indicating the presence of two nearly identically sized pools of QA with different redox potentials or energy levels. A small peak of 21.3°C was also calculated for slr1471-gfp without DCMU, most likely representing a recombination from the S2-P680QA state in the absence of a QB- inhibitor. We hypothesize that electron transfer between QA and QB is altered by the closer energetic level (QA lower to QB). We confirmed the presence of a QB pool emitting at 21.3°C, which would be more pronounced for the fast repair of PSII. Significant photoinhibition in higher plants becomes obvious at high light intensities of 1500 to 2000 μmol·m⁻²·s⁻¹, which is thus widely used as the light intensity for the in vivo analysis of photoinhibition (Trebst et al., 2002; Allakhverdiev et al., 2005; Hakala et al., 2005). Therefore, the growth defect of slr1471-gfp at light intensities of 40 to 80 μmol·m⁻²·s⁻¹ is remarkable. Light sensitivity in slr1471-gfp relates to a decreased repair rate and an increased photodamaging rate of D1, because the altered D1 state in PSII could not be balanced by a higher rate of repair to replenish functional D1.

In vivo labeling of the mutant revealed that integration and assembly of D1 into the thylakoid membrane and PSII were altered, because an unintegrated or incompletely integrated unprocessed pD1 accumulated. This alteration was light-independent, suggesting that defective D1 membrane integration is the primary effect of the GFP fusion to Slr1471p. Several lines of evidence support the idea that pD1 was at maximum only partially integrated in slr1471-gfp cells: (1) defective C-terminal pD1 processing indicated that the essential transfer of the C terminus into the periplasmic space and/or the thylakoid lumen was affected, so at least the last transmembrane domain needs catalysis to integrate properly into the membrane; (2) although pD1 could not be extracted with carbonate from the membrane, the radiolabeled membrane proteins revealed a different protease digestion pattern (Anbudurai et al., 1994; Klinkert et al., 2004); and (3) after BN-PAGE, pD1 was detectable as a protease digestion pattern in vivo analysis of photoinhibition (Trebst et al., 2002; Allakhverdiev et al., 2005; Hakala et al., 2005). Therefore, the growth defect of slr1471-gfp at light intensities of 40 to 80 μmol·m⁻²·s⁻¹ is remarkable. Light sensitivity in slr1471-gfp relates to a decreased repair rate and an increased photodamaging rate of D1, because the altered D1 state in PSII could not be balanced by a higher rate of repair to replenish functional D1.

As a result, D1 turnover is very high and D1 synthesis increases in parallel with light intensity (Andersson and Aro, 2001). The degree of photoinhibition in vivo reflects the level of equilibrium between PSII photodamage and PSII repair. If the repair process is inhibited or too slow relative to the damage rate, photodamaged PSII accumulates, leading to slower or completely stopped growth (Allakhverdiev et al., 2005). At light intensities up to 100 μmol·m⁻²·s⁻¹, the rate of photodamage is balanced by the fast repair of PSII. Significant photoinhibition in higher plants becomes obvious at high light intensities of 1500 to 2000 μmol·m⁻²·s⁻¹, which is thus widely used as the light intensity for the in vivo analysis of photoinhibition (Trebst et al., 2002; Allakhverdiev et al., 2005; Hakala et al., 2005). Therefore, the growth defect of slr1471-gfp at light intensities of 40 to 80 μmol·m⁻²·s⁻¹ is remarkable. Light sensitivity in slr1471-gfp relates to a decreased repair rate and an increased photodamaging rate of D1, because the altered D1 state in PSII could not be balanced by a higher rate of repair to replenish functional D1.

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defined complex, indicating unsppecific aggregation of pD1. Besides pD1, a fully integrated and processed D1 accumulated in slr1471-gfp that assembled into PSII complexes. However, the accumulation of two important PSII assembly intermediates characterized as reaction center complexes containing D1 and D2 was not detectable in the mutant (Klinkert et al., 2004; Komenda et al., 2004). This finding suggests that the assembly pathway of RCs is altered in the mutant or that the biogenesis of RC complexes is the rate-limiting step for PSII assembly in slr1471-gfp.

In slr1471-gfp cells, PSII function is impaired, which was demonstrated by changed redox potentials of the primary and secondary electron acceptors, Qₐ and Qₑ, which are bound to transmembrane helices 4 and 5 of D2 and D1, respectively. Similar changes of the Qₐ⁻ and Qₑ⁻ redox potentials were detected in mutants of the D-de loop between transmembrane helices 4 and 5 of D1 (Mäenpää et al., 1995; Nixon et al., 1995; Minagawa et al., 1999). Therefore, changed redox potentials of Qₐ and Qₑ in slr1471-gfp appeared to be caused by structural changes of D1, although we cannot exclude the possibility that the structure of D2 is also altered in slr1471-gfp. Based on the data for the altered D1 integration and assembly in slr1471-gfp, we speculate that the fusion of GFP to the C terminus of Slr1471p data for the altered D1 integration and assembly in (Sambrook et al., 1989). Plasmid pIGA (Kunert et al., 2000) carrying a All DNA techniques were performed according to standard procedures.

The wild-type strain (generously provided by N. Murata, National Institute of Basic Biology, Okazaki, Japan) and generated mutant strains of Synechocystis sp PCC 6803 were grown photoautotrophically at 30°C under illumination from incandescent lamps at the indicated light intensity (OD730 = 0.05) from precultures grown at a light intensity of 10 μmol m⁻² s⁻¹ and were further incubated for 3 d under light conditions of 20, 40, and 80 μmol m⁻² s⁻¹.

Generation of Synechocystis Mutants
All DNA techniques were performed according to standard procedures (Sambrook et al., 1989). Plasmid pIGA (Kunert et al., 2000) carrying a gfp gene was kindly provided by M. Hagemann (Universität Rostock).

Various portions of a 2.0-kb segment that covers the coding sequence of the slr1471 gene and its flanking regions were amplified by PCR with wild-type genomic DNA as template. Oligonucleotides used were as follows: forward primer M (5’-CATGCTGATGGCAAGTTG-G-3’; corresponding to nucleotides –299 to –280 relative to the AUG of the slr1471 gene) and reverse primer P (5’-CTGACTGAGTTGAGGTCCCCC-3’; corresponding to nucleotides +1155 to +1130 of slr1471) to amplify a 1.5-kb fragment that was cloned into the Natt-KpnI site of pBluescript II KS– (Stratagene), yielding plasmid 5UTR-slr1471_pBS containing the 0.3-kb upstream sequence and coding sequence of the slr1471 gene; forward primer K (5’-CAGTGGGAAT- TCAAGGACCCG-3’; corresponding to nucleotides +538 to +560 of slr1471) and reverse primer T (5’-GACTCTGGAGGGTCTCCC- TTCTTTTAC-3’; corresponding to nucleotides +1152 to +1130 of slr1471) to amplify the 0.6-kb 3’ half of the slr1471 gene, which was cloned into the HindIII-PstI site of pBluescript II KS+, resulting in slr1471_c-pBS; and forward primer 14 (5’-AGTCGTAACCGGGATT- TAAAACTGCTGTTCCAC-3’; corresponding to nucleotides –89 to –60 of slr1472) and reverse primer 15 (5’-AGTCTCTCTCCTTAC- TAACGTCGAGGGCGAATCA-3’; corresponding to nucleotides +495 to +475 of slr1472) to amplify a 0.6-kb fragment covering the sequence containing the stop codon of the slr1471 gene to the stop codon of the slr1472 gene (fragment Kp-slr1472-Kp). For construction of a plasmid used to express the Slr1471-GFP fusion, the gfp gene was amplified with pIGA as template and the forward primer 1 (5’-AGTCGTAACCGGGATT- TAAAAGGAGAAGAACTTTTCAC-3’; corresponding to nucleotides +1 to +26 relative to the AUG of the gfp gene) and reverse primer 2 (5’-GAAGCTAGATCTTCTTTGATGATTTCTACG-3’; corresponding to nucleotides +716 to +693 of the gfp gene), restricted by PstI and Ecl136I, and inserted into the PstI-Smal site of slr1471c_pBS, resulting in plasmid slr1471-gfp_pBS. The 3’ half of the slr1471 gene in 5UTR-slr1471_pBS was removed by digestion with HindIII and BamHI, and the corresponding fragment that had been excised from slr1471-gfp_pBS by HindIII and BglII was inserted, resulting in 5UTR-slr1471-gfp_pBS. The KpnI fragment Kp-slr1472-Kp was inserted into the KpnI site of 5UTR-slr1471-gfp_pBS, resulting in plasmid 5UTR-slr1471-gfp-slr1472_pBS. The Km9 gene cartridge was excised from pUC4K with HincII and inserted into the Smal site located downstream of the slr1471-gfp gene in 5UTR-slr1471-gfp-slr1472_pBS. The resulting plasmid was designated pslr1471-gfp. For construction of a plasmid used to generate a kanamycin-resistant control strain, which carried the Km9 gene cartridge, slr1471-Km9, Wild-type cells of Synechocystis were transformed with the individual plasmids described previously (Tasaki et al., 1996). For selection of mutant cells, kanamycin was included in the medium at 20 μg/mL.

Generation of Polyclonal Anti-Slr1471 Antibodies
A DNA fragment encoding the C terminus of Slr1471p (Arg-117 to Ser-384) was amplified by PCR from wild-type genomic DNA with the primers

5’-CGGGGATCTCCCTTTTCCGA-3’ (corresponding to nucleotides +349 to +368 relative to the AUG) and 5’-TACGGGTCTTTGTCTTCTTTTAC-3’ (corresponding to nucleotides +1152 to +1130).

The fragment was cloned into the TA cloning site of PCR T7/NT-TOPo (Invitrogen) fused to a sequence encoding an N-terminal His6 tag. The resulting plasmid, his6- slr1471_pT7/NT, was transformed into Escherichia coli BL21. Recombinant His6-Slr1471 was purified from 100-mL cultures by two rounds of
Ni²⁺ affinity chromatography purification according to the manufacturer’s instructions (Qiagen). The eluted fractions containing His₆-Slr1471 were concentrated with Biomax-5K centrifugal concentrators (Millipore) and supplemented with SDS to 2% for injection of rabbits. Production of anti-Slr1471 antiserum was examined by protein gel blot analysis (data not shown).

**Spectroscopy and Thermoluminescence Measurements**

Absorption spectra of 1 mL of Synechocystis culture were recorded with a UV-2401 spectrophotometer equipped with an Ulbrichts sphere (ISR-240A) and the supplied software UV-probe using 0.5-nm step width (Shimadzu Deutschland). 77K fluorescence spectroscopy was performed as described (Ossenbühl et al., 2004) using excitation wavelengths of 440 and 570 nm. Emission spectra were recorded with 0.5-nm step width. PAM measurements were performed as described (Ossenbühl et al., 2004). In Synechocystis, a yield of ~0.4 to 0.5 in PAM measurements is attributable to the high amount of PSI and corresponds to the yield of 0.8 measured in higher plants.

Thermoluminescence measurements were performed using a thermoluminescence device together with a ThermoRegulator TR 2000, a dual-modulation kinetic fluorimeter, and the supplied Fluorwin version 3.0 software (Photon Systems Instruments). For thermoluminescence measurements, dark-adapted cells, which were incubated with or without 50 μM DCMU, were frozen at -10°C, and two saturating flashes were given to drive charge separation within PSIi trapped in the S₂ P₆₈₀Q₈Q₅₃⁻ or S₁ P₆₈₀Q₅₃⁻ state. Charge recombination of the trapped charges was then measured as luminescence as a function of temperature (Ducruet, 2003).

**Oxygen Evolution Measurements**

Oxygen evolution measurements were recorded with Chlorolab2 and the supplied Oxylab software (H. Saur Laborbedarf). Synechocystis cells were grown under low (20 μmol-m⁻²-s⁻¹) and high (80 μmol-m⁻²-s⁻¹) light. Cells corresponding to 2 μg of chlorophyll a were suspended in 1 mL of culture medium enriched with 20 μL of 0.1 M NaHCO₃, and were dark-adapted for 2 min. The amount of oxygen consumption and oxygen evolution was then measured in the presence of 20 μmol-m⁻²-s⁻¹ for the low-light-grown cells and 80 μmol-m⁻²-s⁻¹ for the high-light-grown cells.

**In Vivo Labeling of Synechocystis, Gel Electrophoresis, Coimmunoprecipitation, and Protein Gel Blot Analysis**

The synthesis, stability, and assembly of D1 and D2 into PSI were studied by in vivo labeling of Synechocystis cells, coimmunoprecipitation of protein complexes, and gel electrophoresis of total membranes containing thylakoid membranes as described (Klinkert et al., 2004; Komenda et al., 2002). In brief, Synechocystis cells were incubated with [³⁵S]Met for the indicated times and harvested by centrifugation or by purification over MACS columns (Miltenyi Biotec), and purified with Protein A–MacS (Miltenyi Biotec). For gel blot analysis, total membranes of wild type, slr1471-gfp, and slr1471-KmR cells grown at 80 μmol-m⁻²-s⁻¹ corresponding to 3 μg of chlorophyll a were loaded onto a SDS-PAGE device.

**Accession Number**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number BA000022 (Slr1471p).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Resolution of 77K Fluorescence Spectra (Excitation at 440 nm) into Single Components.

**Supplemental Figure 2.** Resolution of 77K Fluorescence Spectra (Excitation at 570 nm) into Single Components.

**Supplemental Figure 3.** PSIi Function Is Impaired in slr1471-gfp.

**Supplemental Figure 4.** PSI Complexes in the Wild Type and slr1471-gfp.

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Friedrich Ossenbühl, Masami Inaba-Sulpice, Jörg Meurer, Jürgen Soll and Lutz A. Eichacker

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